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Effects of Cardiotrophin on Adipocytes*

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Cardiotrophin (CT-1) is a naturally occurring protein member of the interleukin (IL)-6 cytokine family and signals through the gp130/leukemia inhibitory factor receptor (LIFR) heterodimer. The formation of gp130/LIFR complex triggers the auto/trans-phosphorylation of associated Janus kinases, leading to the activation of Janus kinase/STAT and MAPK (ERK1 and -2) signaling pathways. Since adipocytes express both gp130 and LIFR proteins and are responsive to other IL-6 family cytokines, we examined the effects of CT-1 on 3T3-L1 adipocytes. Our studies have shown that CT-1 administration results in a dose- and time-dependent activation and nuclear translocation of STAT1, -3, -5A, and -5B as well as ERK1 and -2. We also confirmed the ability of CT-1 to induce signaling in fat cells *in vivo*. Our studies revealed that neither CT-1 nor ciliary neurotrophic factor treatment affected adipocyte differentiation. However, acute CT-1 treatment caused an increase in SOCS-3 mRNA in adipocytes and a transient decrease in peroxisome proliferator-activated receptor γ (PPAR γ) mRNA that was regulated by the binding of STAT1 to the PPAR γ 2 promoter. The effects of CT-1 on SOCS-3 and PPAR γ mRNA were independent of MAPK activation. Chronic administration of CT-1 to 3T3-L1 adipocytes resulted in a decrease of both fatty acid synthase and insulin receptor substrate-1 protein expression yet did not effect the expression of a variety of other adipocyte proteins. Moreover, chronic CT-1 treatment resulted in the development of insulin resistance as judged by a decrease in insulin-stimulated glucose uptake. In summary, CT-1 is a potent regulator of signaling in adipocytes *in vitro* and *in vivo*, and our current efforts are focused on determining the role of this cardioprotective cytokine on adipocyte physiology.

Cardiotrophin (CT-1),¹ first identified in a screen of a cDNA library derived from the mouse embryoid bodies, has been

shown to support *in vitro* cardiomyocyte survival and hypertrophy. CT-1 is a naturally occurring protein, 200 amino acids long, with a molecular mass of ~21.5 kDa (1). CT-1 mRNA expression has been detected at high levels in the heart, skeletal muscle, prostate, ovaries, and liver, as well as fetal heart, lung, and kidney. Lower amounts have also been detected in the thymus, small intestine, lung, kidney, pancreas, testes, and brain, whereas no expression was observed in the spleen (2). Sequence analyses and structural considerations have shown that CT-1 is a member of the IL-6 cytokine family (2). The members of this family do not share a great deal of primary amino acid sequence homology (14–24%) (3), but they do share common structural features (4) and utilize gp130 signal transducer protein in their receptor complexes (5). Both functional and receptor binding studies in cultured cardiomyocytes have shown that CT-1 signals through the gp130/LIFR heterodimer without the further requirement for the α -subunit (6–8). However, CT-1 signaling in neuronal cells may require an additional 80-kDa α -subunit (45 kDa after N-linked deglycosylation) present in the receptor complexes in addition to gp130 and LIFR (6, 9). These studies have also shown that CT-1 binds the LIFR with affinity equivalent to that of LIF but fails to bind gp130 alone. However, the addition of gp130 enhances its binding to LIFR. From these observations, it has been deduced that CT-1 initially binds LIFR with a low affinity, followed by the recruitment of gp130 into a high affinity binding complex (7). The formation of gp130/LIFR complex triggers the activation and auto/trans-phosphorylation of receptor-associated JAK kinases.

The activation of JAK family kinases leads to the phosphorylation of the receptor subunits, which can then recruit STAT1 and STAT3, leading to their phosphorylation, nuclear translocation, and the ability to regulate gene expression. Phosphorylated receptor subunits can also recruit the Src homology domain 2 adapter Shc, leading to a complex formation with Grb2 and SOS (10). Grb2-SOS complexes can then activate p21^{ras}, leading to the subsequent, cell type-specific, activation of *raf-1*, MEK, and ERK1/2 MAPKs (11, 12).

As previously noted CT-1 was first identified as a factor promoting cardiac myocyte hypertrophy *in vitro* with activity at concentrations (0.1 nM) much lower than other IL-6 family cytokines. Similarly, an *in vivo* chronic administration of CT-1 to rodents resulted in dose-dependent increases in both heart weight and ventricular weight, whereas the total body weight was unaffected (13). These results mimicked those of chronic *in vivo* stimulation of gp130 receptor (14). It has been suggested that this function of CT-1 is governed through its activation of the JAK/STAT pathway (15).

CT-1 has also been shown to reduce the expression of tumor necrosis factor- α in lipopolysaccharide-treated animals (16). Elevated tumor necrosis factor- α levels have been correlated with myocardial infarction and chronic heart failure (17) as

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¹ The abbreviations used are: CT-1, cardiotrophin; JAK, Janus kinase; LIF, leukemia inhibitory factor; LIFR, LIF receptor; MAPK, mitogen-activated protein kinase; STAT, signal transducers and activators of transcription; ERK, extracellular signal-regulated kinase; CNTF, ciliary neurotrophic factor; PPAR, peroxisome proliferator-activated receptor; IRS, insulin receptor substrate; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; FAS, fatty acid synthase.

well as the development of insulin resistance in type 2 diabetes (18). Like other IL-6 cytokine family members, CT-1 can induce liver acute phase response (19). *In vivo* administration of CT-1 has also resulted in hypertrophy of the liver, kidneys, and spleen, atrophy of the thymus, and increasing platelet and red blood cell counts (13). Also, CT-1 supports the long term survival of spinal motor neurons and ciliary ganglion neurons, and the effects of CT-1 on ciliary ganglions mimic those of CNTF (7).

Previous work from our laboratory has shown that CNTF activates JAK/STAT and MAPK (ERK1 and -2) pathways in adipocytes and has several effects on adipocyte physiology (20). Because of its ability to potently activate the JAK/STAT pathway through the gp130/LIFR dimer, we predicted that CT-1 could affect adipocyte physiology and gene expression. Indeed, we have observed that CT-1 activates the JAK/STAT pathway and ERK signaling pathway in fat cells *in vitro* and *in vivo*. Although this gp130 cytokine does not attenuate 3T3-L1 adipocyte differentiation, we have observed that CT-1 decreased fatty acid synthase and IRS-1 protein in mature adipocytes and results in a decrease of insulin-stimulated glucose uptake. In addition, we have shown that CT-1 induces SOCS-3 mRNA and acutely represses PPAR γ expression. The CT-1-induced repression of PPAR γ correlates with binding of STAT 1 to the PPAR γ 2 promoter, and mutation of this site indicates that it contributes to the expression of PPAR γ 2 under basal conditions. We have also observed that these effects of CT-1 are independent of ERK1 and -2. To our knowledge, this is the first study to demonstrate the effects of CT-1 on fat cells. Recently, it has been shown that the circulating levels of CT-1 are increased in the serum of patients with ischemic heart disease and valvular heart disease (21). Since the onset of cardiovascular disease can be associated with obesity/type 2 diabetes (22, 23), the actions of CT-1 in fat may prove to be a relevant link between these two deadly diseases.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium was purchased from Invitrogen. Bovine and fetal bovine sera were purchased from Sigma and Invitrogen, respectively. Rat recombinant CNTF and human recombinant CT-1 were purchased from Calbiochem. Mouse recombinant LIF was purchased from Chemicon International. Insulin and human recombinant growth hormone were purchased from Sigma. U0126 was purchased from Promega. DNase I and Trizol were purchased from Invitrogen. All STAT antibodies were monoclonal IgGs purchased from Transduction Laboratories or polyclonal IgGs purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The highly phosphospecific polyclonal antibodies for STAT1 (Tyr⁷⁰¹), STAT3 (Tyr⁷⁰⁵), and STAT5 (Tyr⁶⁹⁴) were IgGs purchased from BD Transduction Laboratories and Upstate Biotechnology, Inc. (Lake Placid, NY). Sterol regulatory element-binding protein-1 antibody was a rabbit polyclonal IgG, whereas PPAR γ antibody was a mouse monoclonal IgG, both purchased from Santa Cruz Biotechnology. ERK1/ERK2 antibody was a rabbit polyclonal IgG purchased from Santa Cruz Biotechnology. Active ERK antibody was a rabbit polyclonal IgG purchased from Cell Signaling Technology. Akt and FAS antibodies were rabbit polyclonal IgGs purchased from BD Transduction Laboratories. IRS-1 antibody was a polyclonal IgG purchased from Upstate Biotechnology. Horseradish peroxidase-conjugated streptavidin used for detection of ACC was purchased from Pierce. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. An enhanced chemiluminescence kit was purchased from Pierce. Nitrocellulose and Zeta Probe-GT membranes were purchased from Bio-Rad.

Cell Culture—Murine 3T3-L1 preadipocytes were plated and grown to 2 days postconfluence in Dulbecco's modified Eagle's medium with 10% bovine serum. Medium was changed every 48 h. Cells were induced to differentiate by changing the medium to Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, and 1.7 μ M insulin. After 48 h, this medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, and cells were maintained in this medium until utilized for experimentation.

Preparation of Whole Cell Extracts—Monolayers of 3T3-L1 preadipocytes or adipocytes were rinsed with phosphate-buffered saline and then harvested in a nondenaturing buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Igepal CA-630 (Nonidet P-40), 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, and 2 mM sodium vanadate. Samples were extracted for 30 min on ice and centrifuged at 15,000 rpm at 4 °C for 15 min. Supernatants containing whole cell extracts were analyzed for protein content using a BCA kit (Pierce) according to the manufacturer's instructions.

Preparation of Nuclear and Cytosolic Extracts—Cell monolayers were rinsed with phosphate-buffered saline and then harvested in a nuclear homogenization buffer containing 20 mM Tris (pH 7.4), 10 mM NaCl, and 3 mM MgCl₂. Igepal CA-630 (Nonidet P-40) was added to a final concentration of 0.15%, and cells were homogenized with 16 strokes in a Dounce homogenizer. The homogenates were centrifuged at 1500 rpm for 5 min. Supernatants were saved as cytosolic extract, and the nuclear pellets were resuspended in half the volume of nuclear homogenization buffer and were centrifuged as before. The pellet of intact nuclei was resuspended again in half of the original volume of nuclear homogenization buffer and centrifuged again. A small portion of the nuclei was used for trypan blue staining to examine the integrity of the nuclei. The majority of the pellet (intact nuclei) was resuspended in an extraction buffer containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, and 25% glycerol. Nuclei were extracted for 30 min on ice and then placed at room temperature for 10 min. Two hundred units of DNase I was added to each sample, and tubes were inverted and incubated an additional 10 min at room temperature. Finally, the sample was subjected to centrifugation at 15,000 rpm at 4 °C for 30 min. Supernatants containing nuclear extracts were analyzed for protein content, using a BCA protein assay kit (Pierce).

Gel Electrophoresis and Western Blot Analysis—Proteins were separated in 5, 7.5, 10, or 12% polyacrylamide (acrylamide from National Diagnostics) gels containing SDS according to Laemmli (24) and transferred to nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% fat-free milk for 1 h at room temperature. Results were visualized with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence.

RNA Analysis—Total RNA was isolated from cell monolayers with Trizol according to the manufacturer's instructions with minor modifications. For Northern blot analysis, 20 μ g of total RNA was denatured in formamide and electrophoresed through a formaldehyde-agarose gel. The RNA was transferred to Zeta Probe-GT, cross-linked, hybridized, and washed as previously described (18). Probes were labeled by random priming using the Klenow fragment and [α -³²P]dATP.

Rodent Adipose Tissue Isolation—Animals were euthanized by cervical dislocation, and tissues were immediately removed and frozen in liquid nitrogen. Frozen tissues were homogenized in a buffer containing 150 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Igepal CA-630 (Nonidet P-40), 1 μ M phenylmethylsulfonyl fluoride, 1 μ M pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μ M leupeptin, and 2 mM sodium vanadate. Homogenates were centrifuged for 10 min at 5,000 rpm to remove any debris and insoluble material and then analyzed for protein content. All C57BL/6J mice were obtained from a colony at the Pennington Biomedical Research Center. All animal studies were carried out with protocols that were reviewed and approved by institutional animal care and use committees.

Electromobility Shift Assays—Double-stranded oligonucleotides were annealed by heating single-stranded 5' and 3' oligonucleotides in a boiling water bath and gradually cooling to room temperature. The 4 μ g of double-stranded oligonucleotides were 5'-end-labeled with 20 μ Ci of [³²P]dCTP (400–800 Ci/mmol) and with 1 ml each of 5 mM dATP, dTTP, and dGTP with Klenow fragment. The end-labeling reaction was incubated for 15 min at 30 °C and was stopped by adding 1 μ l of 0.5 M EDTA. End-labeled oligonucleotides were purified using a Princeton CENTRISEP column, according to the manufacturer's instructions (Princeton Separations, Inc.). Specific activity of the oligonucleotides was determined by scintillation counting. Nuclear extracts were incubated with the end-labeled oligonucleotides (50,000 cpm/ μ l) for 30 min on ice. The samples were loaded into a prerun (1 h, 100 V at 4 °C) 6% acrylamide/bisacrylamide TBE gel containing 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0. For supershift analysis, nuclear extracts were preincubated with antibody for 1 h at room temperature. For cold competition, the nuclear extracts were incubated with unlabeled oligonucleotide for 15 min on ice prior to incubation with the labeled probe. The gels were run at 20 mA for ~2 h, dried at 80 °C for 1 h under a

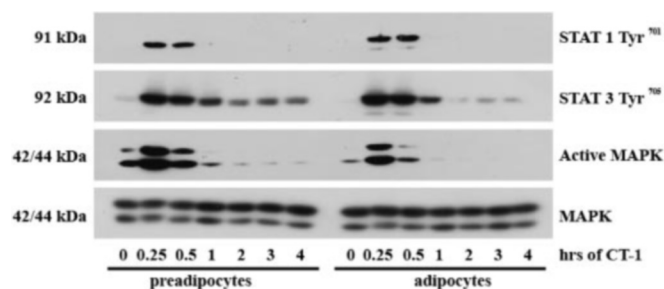


FIG. 1. The effects of acute CT-1 treatment on 3T3-L1 preadipocytes and adipocytes. Whole cell extracts were prepared from confluent undifferentiated preadipocytes and from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times indicated. One hundred twenty-five μ g of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

vacuum, and then exposed to Eastman Kodak Co. BioMax MS film with a Kodak BioMax high energy intensifying screen.

Constructs—The PPAR γ 2 promoter (–609 to +52) luciferase construct was a generous gift of Dr. Jeffrey Gimble, (Pennington Biomedical Research Center). The PPAR γ 2 promoter (–609 to +52) luciferase construct was mutated at positions –217 and –212 within the STAT recognition element using the QuikChange site-directed mutagenesis kit according to the manufacturer's instructions (Stratagene). The following oligonucleotide and corresponding antisense oligonucleotide was used to mutate the STAT recognition element (GAC AAT GTA GCA ACG TTC TCC TCG TAA TGT ACC AAG TC). The mutated bases were altered from T to C and are underlined. The resulting reporter plasmid was termed PPAR γ 2 m217/212 (–609 to +52). The change in sequence was confirmed by sequencing using a Big Dye Terminator Extension Reaction (ABI Prism). The minimum promoter thymidine kinase *Renilla* promoter was obtained from Promega.

Transfections—3T3-L1 preadipocytes were grown to 60% confluence and were transiently transfected with the PPAR γ 2 promoter (–609 to +52)/luciferase construct or the PPAR γ 2 m217/212 (–609 to +52)/luciferase construct and with the thymidine kinase/*Renilla* vector to control for transfection efficiency, using Polyfect transfection reagent according to the manufacturer's instructions (Qiagen). After 48 h of incubation with the Polyfect-DNA solution, cells were treated and then harvested for analysis for firefly luciferase and *Renilla* luciferase activity using the Dual Luciferase Reporter Assay System (Promega). Relative light units were determined by dividing firefly luciferase activity by *Renilla* luciferase activity. Results are given \pm S.D.

Determination of 3 H-Labeled 2-Deoxyglucose Uptake—The assay of 3 H-labeled 2-deoxyglucose uptake was performed as previously described (18). Prior to the assay, fully differentiated 3T3-L1 adipocytes were serum-deprived for 4 h. Uptake measurements were performed in triplicate under conditions where hexose uptake was linear. The results were corrected for nonspecific uptake, and absorption was determined by 3 H-labeled 2-deoxyglucose uptake in the presence of 5 μ M cytochalasin B. Nonspecific uptake and absorption was always less than 10% of the total uptake.

RESULTS

In order to examine the sensitivity of 3T3-L1 cells to CT-1 administration, confluent 3T3-L1 preadipocytes and fully differentiated 3T3-L1 adipocytes were treated with CT-1 (0.20 nM) for the times indicated in Fig. 1. Western blot analysis of cell extracts revealed that both undifferentiated and differentiated 3T3-L1 cells responded to CT-1 treatment in a time-dependent manner. Exposure to CT-1 resulted in a time-dependent activation and tyrosine phosphorylation of STAT1 and -3 as well as activation of MAPK (ERK1 and -2). The magnitudes of the responses were undistinguishable in undifferentiated and differentiated 3T3-L1 cells. However, the tyrosine phosphorylation of STAT3 was sustained for a longer period of time in preadipocytes. The total levels of MAPK are shown as a control for even loading.

The subcellular distribution of STAT proteins following CT-1 treatment was assessed by treating fully differentiated 3T3-L1 adipocytes with CT-1 (0.20 nM) for various periods of time,

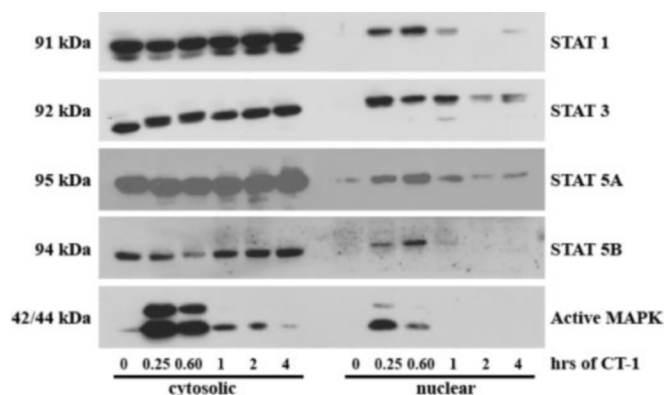


FIG. 2. The effects of acute CT-1 treatment on the activation and nuclear translocation of STAT proteins in 3T3-L1 adipocytes. Cytosolic and nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times indicated. One hundred μ g of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

followed by isolation of cytosolic and nuclear extracts. Western blot analysis of these extracts, shown in Fig. 2, clearly demonstrated that CT-1 treatment results in the nuclear translocation of STAT1, -3, -5A, and -5B as well as the activation of MAPK. For each STAT protein examined, nuclear translocation occurred within 15 min and returned to basal level within 2 h following the cytokine treatment. This pattern is consistent with STAT activation by CNTF and other gp130 cytokines in 3T3-L1 adipocytes (20).

To further examine the ability of CT-1 to activate STATs in 3T3-L1 adipocytes in comparison with other gp130 cytokines, we exposed fully differentiated adipocytes to various doses of CT-1, CNTF, and LIF for 15 min. Western blot analysis of whole cell extracts, shown in Fig. 3, indicated that the activation of STAT1, -3, and -5 and MAPK (ERK1 and -2) by CT-1 is dose-dependent. Moreover, the STAT3 activation was achieved at much lower doses of CT-1 than those required for activation of STAT1 and STAT5. However, as previously shown, CNTF does not activate STAT3 in a dose-dependent manner (20). CNTF treatment also did not result in either STAT1 or STAT5 activation. Treatment with 2.0 nM CNTF resulted in substantially less STAT 3 and MAPK activation when compared with 2.0 nM CT-1. LIF treatment also resulted in a dose-dependent activation of STAT1, -3, and -5 and MAPK in a manner and dose comparable with that of CT-1. The total levels of MAPK are shown as a control for even loading.

To determine if CT-1 could affect signaling pathways in adipose tissue *in vivo*, the effects of this cytokine were examined in 7-week-old C57B1/6J mice given an intraperitoneal injection of CT-1 (0.5 μ g/animal) or vehicle (saline) control and sacrificed after 15 min. Western blot analysis of whole tissue extracts isolated from the epididymal fat pads of the animals, shown in Fig. 4, demonstrated that the four mice injected with CT-1 had a significant increase in levels of active MAPK and tyrosine-phosphorylated STAT1 and -3. However, there was no detectable STAT activation in the four saline-injected mice, although some active MAPK was observed. Also, the activation of STAT5 proteins by CT-1 was not observed under these conditions. The total levels of MAPK are shown as a control for even loading, whereas an extract from 3T3-L1 adipocytes treated with growth hormone was used as a positive control for STAT5 activation (25).

The differentiation of 3T3-L1 preadipocytes into mature adipocytes is governed by a variety of cell signals. Hence, we examined the ability of CT-1 to modulate adipogenesis of

FIG. 3. Dose-dependent effects of CT-1 on 3T3-L1 adipocytes. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes following a 15-min treatment with CT-1, CNTF, or LIF, with the doses indicated in the figure. One hundred fifty μ g of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

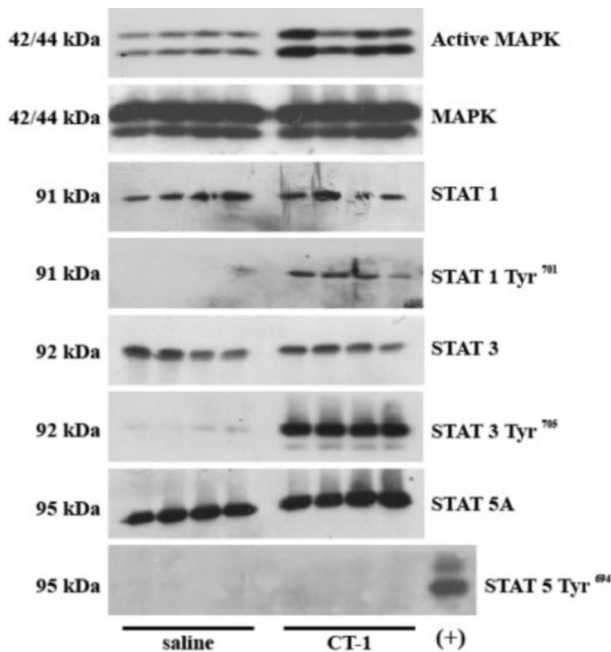
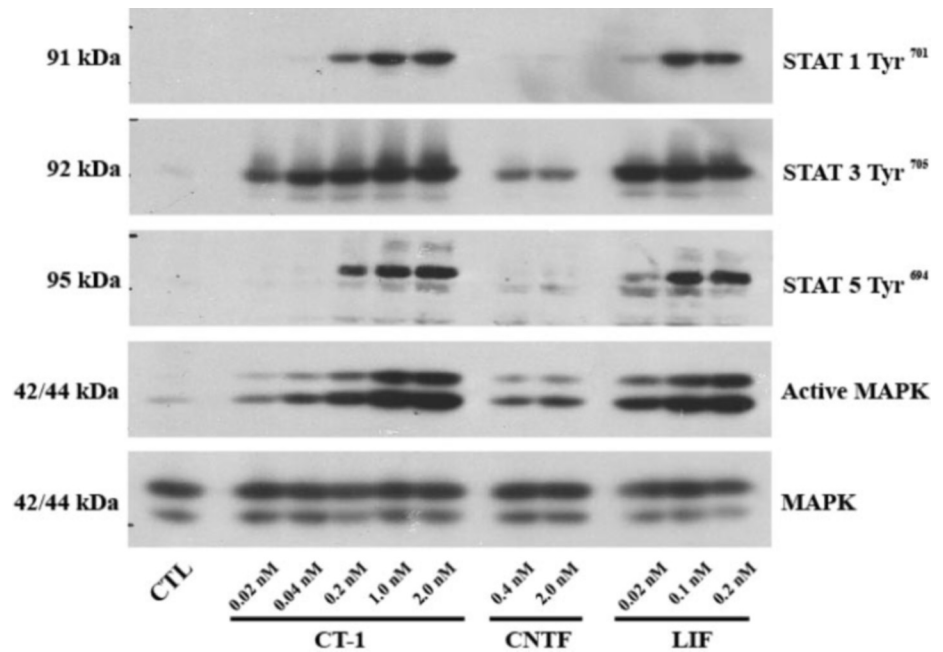


FIG. 4. In vivo effect of acute CT-1 administration in rodents. Seven-week-old male C57B1/6J mice were given an intraperitoneal injection of 0.1 nM CT-1 (0.5 μ g/animal) or vehicle (saline) control. Fifteen min after the injection, the mice were sacrificed, and epididymal fat pads were immediately removed and frozen in liquid nitrogen. One hundred fifty μ g of each tissue extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

3T3-L1 cells. Our results clearly demonstrated that neither CT-1 nor CNTF treatment had any profound effects on adipocyte differentiation, as evident by the unaltered expression levels of various adipocyte proteins including PPAR γ , IRS-1, STAT5A, and ACC (data not shown). Since CT-1 did not affect the adipogenesis of 3T3-L1 cells, we examined the effects of this cytokine on the expression of genes in fully differentiated 3T3-L1 adipocytes. Since this cytokine is a potent STAT activator, both *in vitro* and *in vivo* (Figs. 2 and 4), we hypothesized that activated STATs would modulate transcription in fat cells. Serum-deprived, fully differentiated 3T3-L1 adipocytes were treated with CT-1 (0.20 nM) or CNTF (0.45 nM) for various

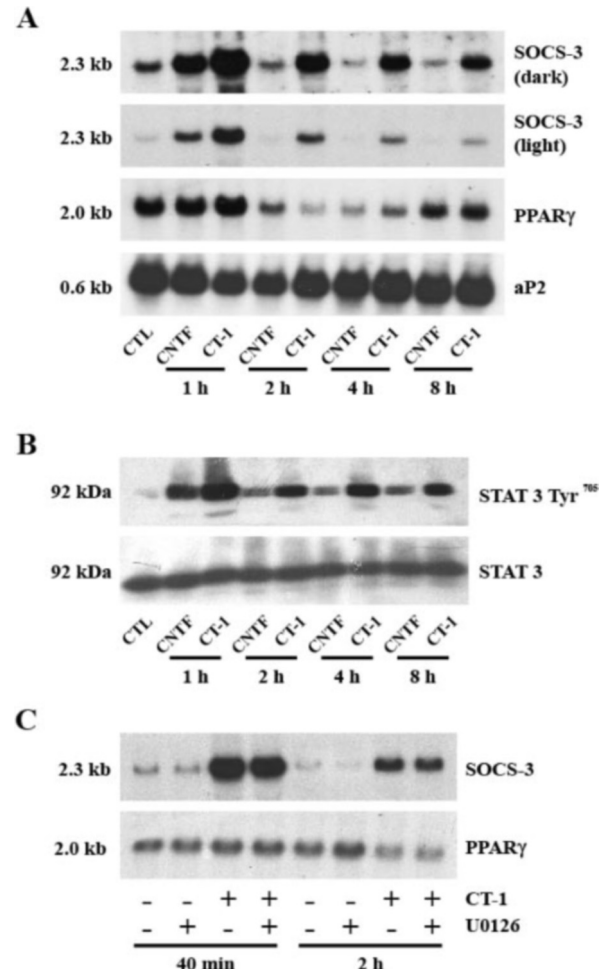
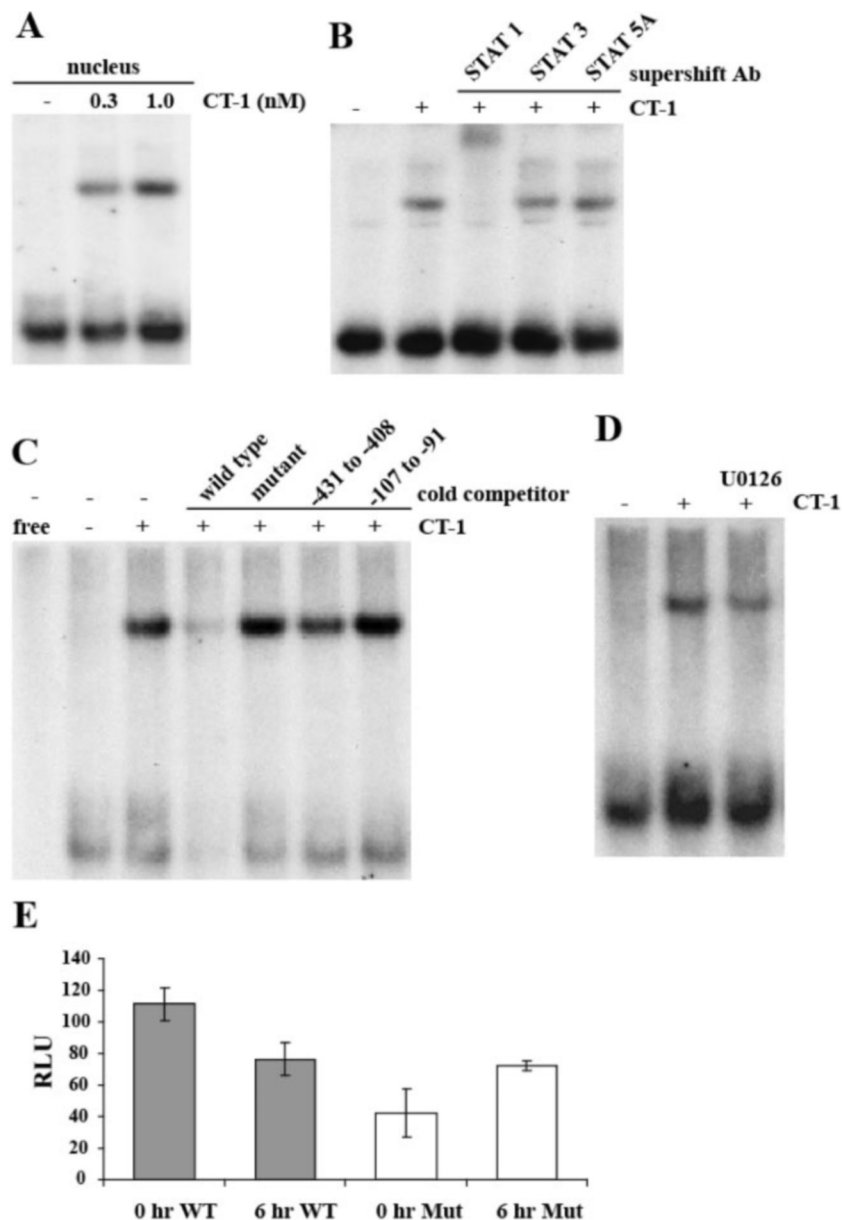


FIG. 5. The effects of acute CT-1 and CNTF administration on the expression of adipocyte proteins. Total RNA (A and C) or whole cell extracts (B) were isolated from fully differentiated 3T3-L1 adipocytes following a treatment with CT-1 (0.2 nM), CNTF (0.45 nM), or U0126 (5.0 μ M) for the times shown. Twenty μ g of each total RNA was electrophoresed, transferred to nylon, and subjected to Northern blot analysis. One hundred μ g of each whole cell extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis.

FIG. 6. CT-1 induces STAT1 binding to the -221 to -207 element of the PPAR γ 2 promoter *in vitro*. A, nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated or treated with the indicated dose of CT-1 for 15 min. The extracts were incubated with a 32 P-labeled oligonucleotide of the -221 to -207 region of the PPAR γ 2 promoter for analysis by an electromobility shift assay. B, nuclear extracts were prepared from 3T3-L1 adipocytes that were untreated or treated with 0.3 nM CT-1 for 15 min. The extracts were preincubated with antibodies for STAT1, -3, or -5A for supershift analysis. The extracts were then incubated with the 32 P-labeled -221 to -207 oligonucleotide. C, prior to incubation with the 32 P-labeled -221 to -207 oligonucleotide, nuclear extracts from CT-1-treated 3T3-L1 adipocytes were incubated with unlabeled oligonucleotides of the -221 to -207 sequence from the PPAR γ 2 promoter, a mutated sequence of the -221 to -207 element, the -431 to -408 sequence of the PPAR γ 2 promoter, or the -107 to -91 sequence from the PPAR γ 2 promoter. D, 3T3-L1 adipocytes were pretreated with U0126 for 45 min and were then treated for 15 min with 0.3 nM CT-1. Nuclear extracts from these cells were then incubated with the 32 P-labeled -221 to -207 oligonucleotide for electromobility shift analysis. E, 3T3-L1 preadipocytes were transiently transfected with either the PPAR γ 2 promoter (-609 to +52)/luciferase reporter wild-type (WT) construct or with the PPAR γ 2 m217/212 (-609 to +52)/luciferase reporter mutant (Mut) construct. The cells were also transfected with the thymidine kinase/*Renilla* construct to normalize for transfection variability. After 48 h, the cells were untreated or treated with 0.4 nM CT-1 for 6 h and then were harvested for analysis. Relative light units (RLU) was calculated by dividing the luciferase reporter activity by the *Renilla* luciferase activity. Results are shown as mean \pm S.D.



times, indicated in Fig. 5. Total RNA and whole cell extracts were collected following the treatment. As shown in Fig. 5A, both CT-1 and CNTF administration resulted in a rapid induction of SOCS-3 mRNA levels. However, this up-regulation was very transient following CNTF treatment, whereas CT-1 treatment, at a dose lower than that of CNTF, resulted in a more sustained induction of SOCS-3 mRNA. Both cytokines also resulted in a transient down-regulation of PPAR γ mRNA, which was more evident following a 2-h CT-1 treatment. The levels of *aP2* mRNA were unchanged. The efficacy of the cytokines in this experiment was demonstrated by their ability to induce STAT3 phosphorylation (Fig. 5B). Since CT-1 is a potent activator of the MAPK pathway, we set out to determine whether the repression of PPAR γ mRNA or induction of SOCS-3 mRNA induced by CT-1 was mediated through this pathway by pretreating 3T3-L1 adipocytes with U0126, a MEK inhibitor. The Northern blot analysis in Fig. 5C clearly demonstrates that both SOCS-3 mRNA up-regulation and PPAR γ mRNA down-regulation are not dependent on the activity of ERK1 and -2, because the addition of U0126 failed to prevent modulation of mRNA levels by CT-1. The efficacy of U0126 was

confirmed by the loss of MAPK (ERK1 and -2) activation following an acute CT-1 treatment (data not shown).

Previous work from our laboratory has shown that down-regulation of PPAR γ by interferon- γ can occur at the level of transcription and may be mediated by STAT1 binding to the -221 to -207 element of the PPAR γ 2 promoter (26). Therefore, we examined the ability of CT-1-activated STAT1 proteins to bind the -221 to -207 element of the PPAR γ 2 promoter. To analyze the binding of protein complexes to the -221 to -207 oligonucleotide, electromobility shift assays were performed with nuclear extracts from 3T3-L1 adipocytes acutely treated with CT-1 for 15 min. As shown in Fig. 6A, two protein complexes that shifted the -221 to -207 oligonucleotide were detected, but only the slower migrating band was induced with CT-1 treatment in a dose-dependent manner (lane 2). In order to determine whether the CT-1-induced protein complex contained STAT proteins, a supershift analysis was performed, using antibodies for STAT1, -3, and -5A. The STAT1 antibody fully supershifted the CT-1-induced protein complex (lane 3); however, the STAT3 and -5A antibodies did not affect the mobility of the protein complex, as shown in Fig. 6B (lanes 4

and 5), indicating that the protein complex may be a STAT1 homodimer. Specificity of binding to the labeled oligonucleotide (–221 to –207) is shown in Fig. 6C. Binding of the CT-1-activated protein complex was competed with an excess of the unlabeled wild type oligonucleotide (lane 4) but not with a mutant oligonucleotide (lane 5) nor with oligonucleotides corresponding to two other sites within the PPAR γ 2 promoter that bear resemblance to the STAT consensus sequence TTC-NNNGAA (lanes 6 and 7). Since serine phosphorylation of STATs can modulate the transcriptional activity of these proteins (27), we examined the binding of CT-1-activated STAT 1 to the –221 to –207 element of the PPAR γ 2 promoter following a pretreatment with U0126. The binding of STAT1 to the –221 to –207 oligonucleotide was moderately inhibited by the MEK inhibitor, shown in Fig. 6D, indicating a possible role for the Ras/MAPK pathway in regulating STAT1 binding to the PPAR γ 2 promoter. Yet, the ability of CT-1 to induce STAT 1 binding to the PPAR γ 2 promoter was not dependent on active MAPK (ERK1 and -2). To assess the importance of this STAT1 site in the CT-1-induced modulation of PPAR γ , we transiently transfected 3T3-L1 preadipocytes with a wild type (WT in Fig. 6) PPAR γ 2 promoter (–609 to +52)/luciferase construct or with mutant PPAR γ 2 promoter (*Mut*) construct that had two nucleotides altered within the –221 to –207 region (PPAR γ 2 m217/212 (–609 to +52)/luciferase). The results in Fig. 6E demonstrate that a 6-h treatment with CT-1 resulted in a 30% decrease in luciferase activity of the wild type construct, indicating decreased transcription of the PPAR γ 2 promoter by this gp130 cytokine. In addition, transfection with the mutant construct revealed lower basal level of luciferase activity, indicating that this STAT1 binding site contributes to the expression levels of this reporter/construct. Interestingly, a 6-h CT-1 treatment of preadipocytes containing the mutant construct resulted in an up-regulation of the promoter. Since the basal levels of expression of the mutant construct are reduced, it cannot be determined whether the mutated bases within the –221 to –207 region are critical for the CT-1-induced down-regulation.

To assess the effect of chronic CT-1 treatment on 3T3-L1 adipocytes, fully differentiated adipocytes were exposed to CT-1 over a time course of 96 h. A fresh bolus of CT-1 was added to the cells every 24 h. As shown in Fig. 7, chronic administration of CT-1 resulted in decreased protein levels of FAS and IRS-1 after 72 and 96 h of treatment. The levels of ACC, the 67-kDa form of sterol regulatory element-binding protein-1, and Akt were unchanged. In addition, there were no significant effects on the levels of STAT1, -3, and -5A. Interestingly, CT-1 administration resulted in a transient down-regulation of PPAR γ protein following a 24-h CT-1 treatment. However, after 48 h, PPAR γ levels had returned to basal levels, and additional CT-1 treatments did not result in decreased PPAR γ following chronic (48–96 h) CT-1 treatment.

Since we observed a decrease in IRS-1 protein levels following a chronic CT-1 treatment of 3T3-L1 adipocytes, we examined the effects of CT-1 on insulin-stimulated glucose uptake in these cells. As shown in Fig. 8A, a 10-min treatment with insulin results in a nearly 6-fold increase in glucose uptake in fully differentiated 3T3-L1 adipocytes. A 1-h pretreatment with CT-1 did not cause a statistically significant inhibition of insulin-stimulated glucose uptake. However, we observed a decrease in insulin-stimulated glucose uptake following a 24-h and a 96-h pretreatment with CT-1. Specifically, the 96-h CT-1 pretreatment resulted in a statistically significant 4-fold decrease in insulin-stimulated glucose uptake, as compared with the control, that was largely due to a statistically significant increase in basal glucose uptake, as indicated in Fig. 8B.

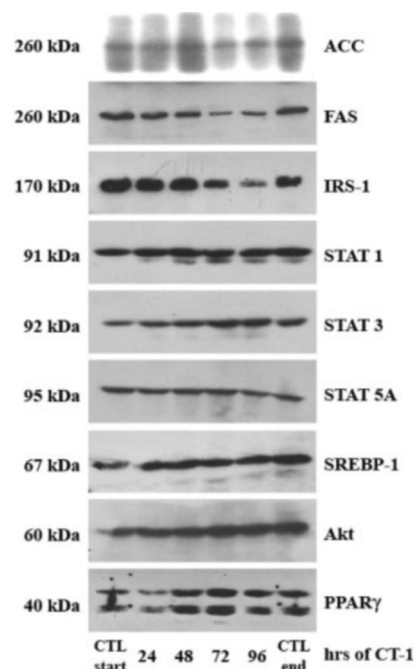


FIG. 7. The effects of chronic CT-1 administration on the expression of adipocyte proteins. Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes following a treatment with 0.2 nM CT-1 for the times shown. One hundred μ g of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. CTL, control.

DISCUSSION

CT-1 is a newly identified member of the gp130 cytokine family that signals via a receptor complex composed of gp130 and LIFR signal transducer proteins. Recent work from our laboratory has shown that another member of the gp130 cytokine family, CNTF, can activate several signaling pathways and regulate protein expression in 3T3-L1 adipocytes (20). These effects are likely to be induced by utilizing only gp130 and LIFR as signal transducers, since the specific CNTF receptor, CNTF receptor α , is not expressed in fat cells *in vitro* (20). Since both IL-6 and CNTF have effects on body weight regulation (28, 29), we have investigated the actions of CT-1 *in vitro* and *in vivo* to determine whether this gp130 cytokine can induce similar signaling effects in fat cells and regulate adipocyte gene expression.

Our results demonstrated that administration of CT-1 to both preadipocytes and 3T3-L1 adipocytes resulted in similar downstream responses. Interestingly, and in contrast to CNTF, CT-1 activated STAT1 and -3, as well as ERK1 and -2 MAPKs in both cell types, and the responses were of identical magnitudes but slightly more sustained in preadipocytes. Since the levels of gp130 remain fairly unaffected by differentiation, and LIFR is expressed by mature adipocytes, both preadipocytes and mature adipocytes have a significant population of these receptors on their cell surfaces. Therefore, both cell types are likely to be activated by CT-1. The attenuation of the response duration in adipocytes, as compared with preadipocytes, could be attributed to the slight loss of LIFR expression that occurs during adipocyte differentiation (30).

Further investigation of CT-1 actions in adipocytes revealed that CT-1 was also capable of activating STAT5A and -5B *in vitro*, in addition to STAT1 and -3. STAT5A and -5B in 3T3-L1 adipocytes were not activated by CNTF or other gp130 cytokines. Hence, their activation by CT-1 presents a rather novel finding and may, upon further investigation, prove to be a mechanism for the specificity of this cytokine's action. The

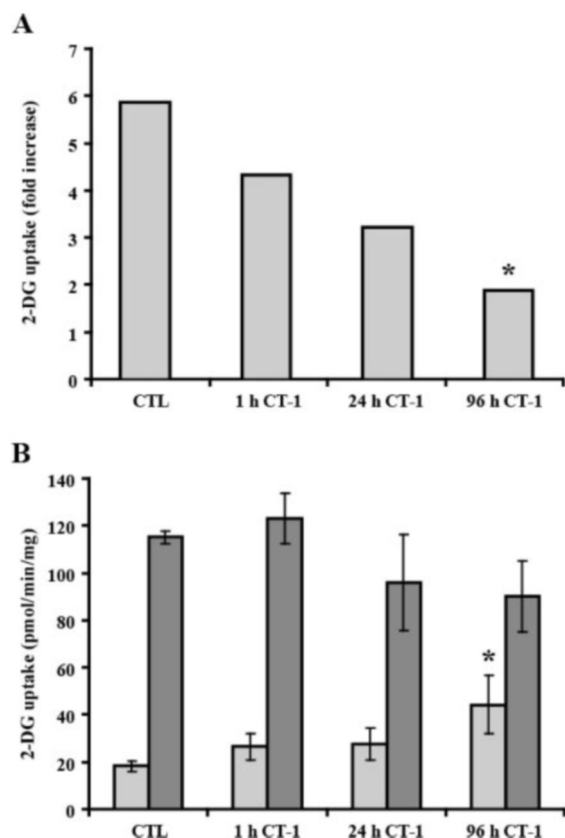


FIG. 8. **The effects of CT-1 administration on insulin-stimulated glucose uptake.** Serum-deprived, fully differentiated 3T3-L1 adipocytes were incubated with 0.2 nM CT-1 for the periods indicated in the figure. Cells were then exposed to 50 nM insulin or saline control (CTL) for 10 min. Glucose uptake was measured as described under "Experimental Procedures." A, values shown represent the -fold increase in glucose uptake, over basal uptake level, following the insulin treatment (*, $p = 0.023$, Student's t test). B, values shown represent the mean \pm S.E. (light bars, saline control; dark bars, insulin treatment) of triplicate determinations from two independent experiments (*, $p = 0.019$, Student's t test).

activation of these proteins was confirmed through their detection in the nuclear fraction from the cells following cytokine treatment. STAT proteins translocated to the nucleus within 15 min after the treatment and were mostly absent within 2 h. These parameters are consistent with STAT activation by other cytokines in adipocytes (31). These data also indicate that STATs activated by CT-1 are not only tyrosine-phosphorylated but also translocate to the nucleus, where they can act as transcription factors.

Interestingly, CNTF cannot activate STAT3 and ERK1 and -2 in a dose-dependent manner in adipocytes (20). However, adipocytes treated with a range of CT-1 doses activated STAT1, -3, and -5 as well as ERK1 and -2 in a dose-dependent manner. Activation of these proteins by CT-1 also occurred at much lower doses and with higher magnitude of activation when compared with that of CNTF. Once again, we hypothesize that this response is due to the abundance of gp130 and LIFR in adipocytes. Unlike CNTF, which has a limited affinity for gp130/LIFR dimer in the absence of CNTF receptor α , CT-1 is not limited by the absence of the cytokine-specific receptor component and can bind gp130/LIFR dimer with a very high affinity (7). LIF, a gp130 cytokine which also utilizes the gp130/LIFR dimer to transduce its signals, is capable of activating an identical profile of adipocyte STATs as CT-1 and also does so in a dose-dependent manner. Thus, taking into account the results discussed so far, one can hypothesize that the availability of receptor component proteins can significantly affect the mag-

nitude, duration, variety, specificity, and dose dependence of gp130 cytokine response in fat cells.

Native adipocytes also express both gp130 and LIFR proteins. Hence, we investigated the *in vivo* effect of CT-1 administration on adipose tissue of C57B1/6J mice. We observed that acute CT-1 was able to activate STAT1 and -3, as well as ERK1 and -2, in the epididymal fat pads of these mice, mimicking the *in vitro* effects we observed in 3T3-L1 adipocytes. However, the dose administered (0.1 nM) was not sufficient to induce the activation of STAT5. This is consistent with the dose dependence results from the *in vitro* studies. Although further studies will be necessary to determine whether CT-1 can activate STAT5 at higher doses *in vivo*, it is still quite exciting to discover that this cytokine directly affects fat tissue and may potentially have biological roles in native adipocyte function.

Adipocyte differentiation is marked by changes in the expression of several adipocyte-specific proteins. Since CT-1 and CNTF both activate the JAK/STAT pathway in 3T3-L1 cells, we examined the ability of these cytokines to regulate adipogenesis. Unlike tumor necrosis factor- α or interferon- γ , the presence of CT-1 or CNTF did not inhibit adipocyte differentiation. These studies suggest that the anti-obesity effects of CNTF are not mediated by the inhibition of fat cell differentiation. Interestingly, both cytokines induced a decrease in the expression of FAS, which we have previously observed with chronic CNTF treatment in adipocytes (20). Since CT-1 administration to differentiating 3T3-L1 cells did not affect overall lipid accumulation (data not shown), we cannot be certain whether CT-1 could potentially affect lipid biosynthesis in adipocytes. However, in mature adipocytes, both gp130 cytokines resulted in a transient decrease in PPAR γ expression.

Although CT-1 and CNTF did not affect adipocyte differentiation, we further investigated the acute effects of these cytokines on fully differentiated 3T3-L1 adipocytes. Acute treatment of 3T3-L1 adipocytes with CNTF and CT-1 resulted in a significant induction of SOCS-3 mRNA levels. Within 1 h, the levels returned to basal levels in the CNTF-treated cells but remained elevated even after 8 h in CT-1-treated adipocytes. However, this finding was not very surprising due to the fact that SOCS-3 mRNA levels are regulated by activated STATs (32), and we have shown that the activation of STATs by CT-1, especially the activation of STAT3, is temporally prolonged, compared with activation by CNTF. Another interesting finding is that both cytokines, especially CT-1, resulted in a transient decrease of PPAR γ mRNA levels. This is consistent with the transient decrease in the PPAR γ protein we observed following a 24-h CT-1 treatment (Fig. 8). Other studies have shown that STATs activated by interferon- γ , tumor necrosis factor- α , IL-1, and IL-6, have the ability to down-regulate PPAR γ mRNA levels (33, 34). Since CT-1 is predominantly known for its role as an activator of the JAK/STAT and MAPK pathways, we were interested in determining which of these pathways was involved in the induction of SOCS-3 and the attenuation of PPAR γ mRNA levels. When the MAPK pathway was inhibited by using the specific MEK inhibitor U0126, we still observed an increase in SOCS-3 mRNA and a decrease in PPAR γ mRNA levels following CT-1 treatment. Hence, our studies suggest that the CT-1-induced regulation of SOCS-3 and PPAR γ mRNA levels is independent of the MAPK (ERK1 and -2) pathway.

Since previous studies from our laboratory have demonstrated that STAT1 activated by interferon- γ down-regulates the expression of PPAR γ via the -221 to -207 site within the promoter (26), we investigated the ability of STAT1 activated by CT-1 to bind this promoter element. Our findings clearly demonstrate that CT-1 induces STAT1 binding to the PPAR γ 2

promoter element and that this binding is very specific and may play a role in the CT-1-induced repression of PPAR γ 2 promoter activity. We also observed that this regulation is independent of the actions of MAPK. Together with our results demonstrating that CT-1 decreases PPAR γ mRNA levels (Fig. 7A) and results in a transient decrease in PPAR γ protein expression (Fig. 8), we conclude that a single exposure of adipocytes to CT-1 results in a repression of PPAR γ transcription that is independent of MAPK activity.

In light of our results from the acute CT-1 administration, we also examined the effects of chronic CT-1 treatment in 3T3-L1 adipocytes. Upon 96 h of treatment, CT-1 resulted in a decrease of FAS and IRS-1 protein levels. CNTF had an identical effect on FAS but an opposite effect on IRS-1 (20). The levels of STATs, Akt, and sterol regulatory element-binding protein-1 remained constant throughout the treatment, unlike that of PPAR γ . CT-1 first induced a transient decrease in the protein levels, consistent with the decrease in mRNA levels we observed in the acute treatment. However, PPAR γ protein expression was then transiently increased before returning to the basal level. The ability of CT-1 to regulate PPAR γ may prove to be interesting in its relation to effecting adipocytes, particularly since CT-1 did not inhibit PPAR γ expression in differentiating cells. More importantly, the down-regulation of IRS-1 expression by CT-1 could be a possible marker of impaired insulin sensitivity in CT-1-treated adipocytes. Our observation that chronic (96 h) CT-1 treatment of fully differentiated 3T3-L1 adipocytes results in a statistically significant decrease in insulin-stimulated glucose uptake (Fig. 8A) supports our hypothesis that CT-1 may be a mediator of impaired insulin sensitivity. However, further work should be done to address these results, particularly since our findings (Fig. 8B) indicate that a 96-h CT-1 treatment leads to a statistically significant increase in basal glucose uptake, rather than a notable decrease in insulin-stimulated uptake. We hypothesize that the increase in basal uptake may be due to increased GLUT1 expression. Currently, there are no reports of increased circulating levels of CT-1 in conditions of insulin resistance, yet we hypothesize that the modulation of IRS-1 by CT-1 may serve as a model of insulin resistance to study the effect of elevated circulating levels of CT-1 in the serum of patients with ischemic heart disease and valvular heart disease (21). Clinical aspects of these diseases are tightly linked to obesity/type 2 diabetes (22, 23), whereas the principal cause of diabetes mortality is cardiovascular disease (35). Therefore, CT-1 may act as a link between obesity-related complications and cardiovascular disease.

In summary, we have observed that CT-1 acts as a potent activator of both JAK/STAT and MAPK pathways in both preadipocytes and 3T3-L1 adipocytes as well as in rodent fat pads *in vivo*. Unlike CNTF, CT-1 activation of these pathways is more robust, dose-dependent, and sustained. This is also the first report of STAT5 activation by a gp130 cytokine in 3T3-L1 adipocytes. Although neither CNTF nor CT-1 had any significant effects on adipocyte differentiation, both cytokines induced transient changes in SOCS-3 and PPAR γ mRNA and protein levels in adipocytes. Since other gp130 cytokines, such as leptin and CNTF, have been implied as potential mediators of various aspects of obesity/type 2 diabetes, it would be inter-

esting to further investigate the actions of CT-1 in this area. However, the strongest focus should be placed on measuring the circulating levels of CT-1 in obesity/type 2 diabetes patients and the effects of this cytokine on insulin-sensitive tissues.

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